Incubation under fluid dynamic conditions markedly improves the structural preservation *in vitro* of explanted skeletal muscles

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Abstract

Explanted organs and tissues represent suitable experimental systems mimicking the functional and structural complexity of the living organism, with positive ethical and economic impact on research activities. However, their preservation in culture is generally limited, thus hindering their application as experimental models for biomedical research. In the present study, we investigated the potential of an innovative fluid dynamic culture system to improve the structural preservation *in vitro* of explanted mouse skeletal muscles (soleus). We used light and transmission electron microscopy to compare the morphological features of muscles maintained either in multiwell plates under conventional conditions or in a bioreactor mimicking the flow of physiological fluids. Our results demonstrate that fluid dynamic conditions markedly slowed the progressive structural deterioration of the muscle tissue occurring during the permanence in the culture medium, prolonging the preservation of some organelles such as mitochondria up to 48 h.

Introduction

During the last decades, the adoption by scientists of the "3 Rs" principles (Replacement, Reduction and Refinement)[1] has led to progressive reduction of animals used for scientific experimentation. In fact, this set of ethical principles proposes that every effort should be made to *Replace* animals with non-sentient alternatives, to *Reduce* to a minimum the number of animals used in experiments, and to *Refine* the experiments so as to cause the least pain and distress. However, research aimed at setting up novel therapeutic and/or diagnostic strategies in human or veterinary medicine, or at performing efficacy/safety evaluations

mental models able to mimic efficiently the complex structural and functional features of living organisms or organs. Unfortunately, the currently available *in vitro* systems, from the conventional 2D cell cultures to the 3D co-cultures, can be suitably used only in basic or early-phase applied research because they are not able to reproduce the systemic milieu. On the other hand, the promising technology of microfluidic organs-on-chips, intended to simulate levels of tissue and organ functionality not possible with 2D or 3D culture systems,[2] is still far from reliably mimicking tissue and organ physiology.

It is therefore necessary to develop alternative experimental models characterized by a functional and structural complexity similar to the living organism. In this view, explanted organs and tissues could represent a suitable and relatively easy solution: surgical and bioptic explants from human or animal subjects could be used for scientific purposes thus drastically reducing tests on laboratory animals. However, the preservation in culture of explanted organs and tissues is generally limited, thus hindering their application as experimental models for biomedical research, especially for long-term studies.

Skeletal muscle is a highly differentiated organ with a complex cytoarchitecture whose maintenance *in vitro* has been scarcely explored. In the present study, we investigated the potential of an innovative fluid dynamic *in vitro* system to improve the structural preservation of explanted mouse skeletal muscles. We used light and transmission electron microscopy to compare the morphological features of muscles maintained either in multiwell plates under conventional conditions or in a bioreactor mimicking the flow of physiological fluids.

Materials and Methods

Muscle isolation and incubation

Soleus muscles were explanted from healthy 3-month-old male Balb/c mice sacrificed in the frame of a research project approved by the Italian Ministry of Health (protocol code: ZA/14/18). The mice were previously anaesthetized with an overdose of isoflurane using a pre-anesthesia chamber and then we proceeded with the cervical dislocation. The soleus muscle was isolated and excised from each paw, and four muscles per experimental group were used (see Figure 1 for the experimental plan).

Freshly excised muscles were rapidly washed in pre-warmed culture medium (see below) at 37°C and then maintained in an incubator at 37°C in a 5% CO_2 humidified atmosphere for 6 h, 24 h and 48 h, under either conventional or fluid dynamic condition (the bioreactor can be maintained into the incubator). The culture medium was composed of medium 199 (Gibco, Waltham, MA, USA) supplemented with 5.5 mM glucose, 2.54 mM $CaCl_2$ (Merk, Kenilworth, NJ, USA), 25 mM NaHCO3, 0.6 nM insulin (Sigma-Aldrich, St. Louis, MO, USA), 0.1% BSA (Gibco), 200 uU/mL penicillin-streptomycin (Gibco) and 0.5% Amphotericin B (Gibco), according to.[3]

For conventional culture condition, muscles were incubated in 12-multiwell plastic plates containing 2 mL of medium each: during the first incubation day, the medium was replaced every 6 h while in the following days it was changed every 4 h; in addition, the plates were gently shaken manually every 30 min, with the exclusion of the overnight incubation.

For fluid dynamic culture condition, a LiveFlow bioreactor compatible with the incubator environment (IV-Tech, Massarosa, LU, Italy) was used. In detail, muscles were placed in cell culture chambers (LiveBox1) containing 1.5 mL of the same medium used for conventional condition; four chambers were joined in series in a fluidic circuit connected to a 15 mL-mixing chamber; a flow rate of 300 µL/min was applied. Similar to conventional condition,

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the first day of incubation the medium was replaced every 6 h, while for the following days it was changed every 4 h.

As controls for time 0, some muscle samples were processed for light and transmission electron microscopy (see below) immediately after excision.

Tissue processing for microscopy analysis

To evaluate the integrity of the explanted muscles at each incubation times, the samples were processed for light and transmission electron microscopy. For light microscopy, the muscles were immersed in isopentane precooled in liquid nitrogen for 30 s to allow the complete freezing, then they were kept at -80°C. For sectioning, the samples were embedded in OCT, and 10 µm-thick sections were cut in a cryostat and collected on glass slides. For morphological observations, cryosections were hydrated for 5 min in PBS, dipped in Mayer's hematoxylin (Sigma-Aldrich) for 90 s, rinsed with tap H_2O for 5 min, stained with eosin (Sigma-Aldrich) for 30 s, dehydrated in graded ethanols, cleared in xylene, and mounted with Entellan (Sigma-Aldrich). The sections were observed with an Olympus BX51 microscope equipped with a 40x objective lens; micrographs were taken with an Olympus Camedia 5050 digital camera.

For transmission electron microscopy, muscles were fixed with 2% (v/v) paraformaldehyde and 2,5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 2 h, post-fixed with 1% OsO_4 and 1.5% potassium ferrocyanide at room temperature for 1h, dehydrated with acetone and embedded in Epon. Ultrathin sections were placed on copper grids, stained with lead citrate and observed in a Philips Morgagni transmission electron microscope operating at 80 KV and equipped with a Megaview II camera for digital image acquisition.

Results and Discussion

In this study, soleus muscles explanted from mice were maintained in culture under either conventional (multiwell plates) or fluid dynamic (bioreactor) conditions, and their structural preservation at increasing incubation times was compared by using light and transmission electron microscopy, in order to analyze both the histological organization and the fine morphology of myofibre components. Cryofixation was applied due to the potential of these samples

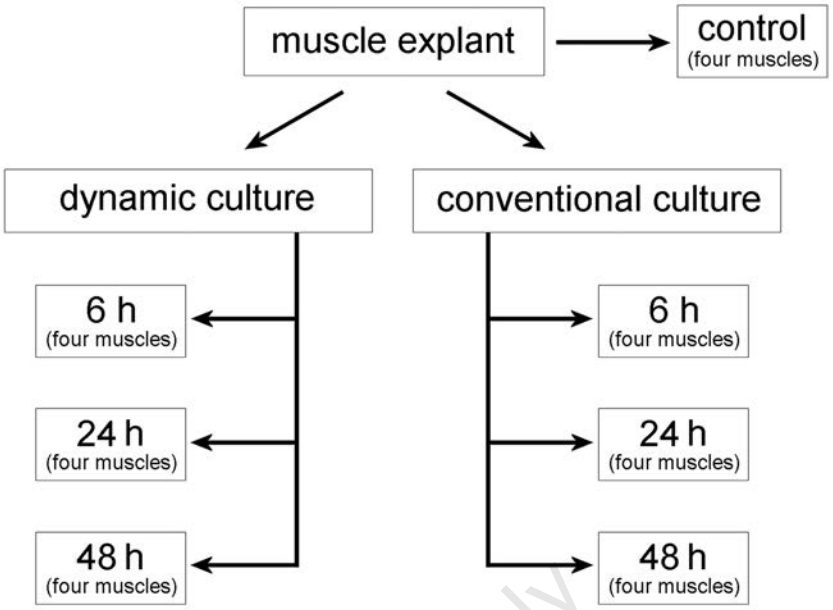

Figure 1. Graphical representation of the experimental plan.

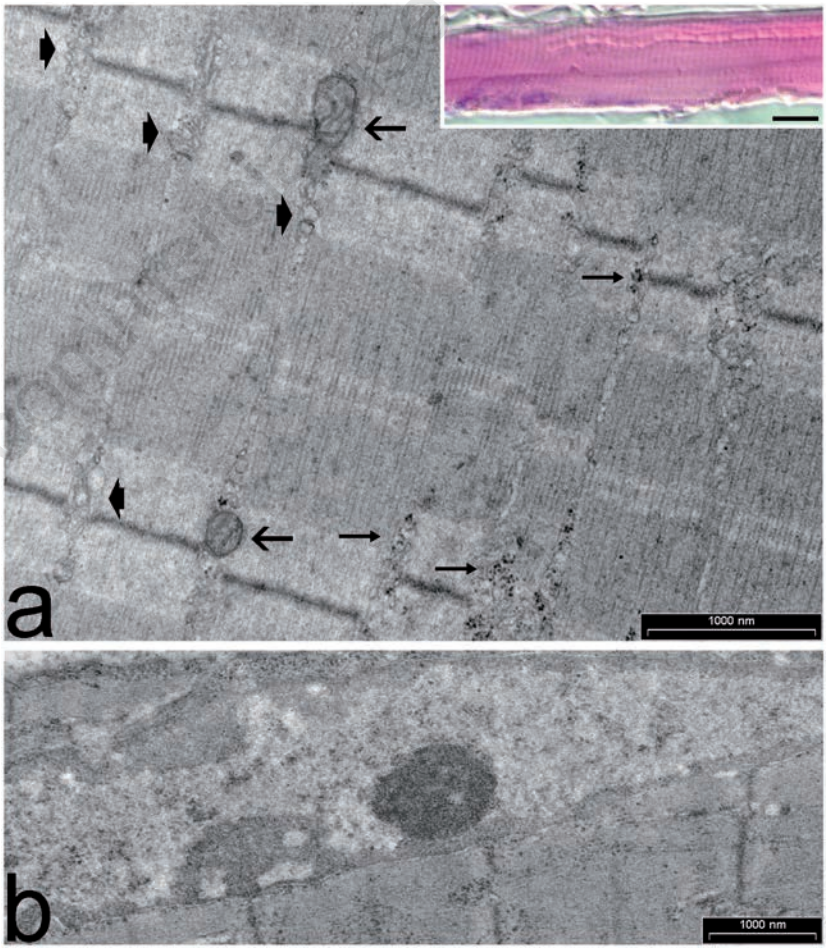

Figure 2. Soleus muscle, control samples. a) The myofibre shows the typical transverse banding and numerus subsarcolemmal nuclei (inset). In the sarcoplasm, the myofilaments are arranged to form sarcomeres, and mitochondria (arrows) and glycogen granules (thin arrows) occur between myofibrils; note the sarcoplasmic reticulum elements (arrowheads). Scale bars: 1000 nm; inset scale bar: 10 µm. b) The myonucleus shows condensed chromatin clumps mainly located at its periphery, and one condensed nucleolus.

to be used not only for morphological analyses (as in the present study), but also for histochemistry and immunohistochemistry at bright field and fluorescence microscopy; this represents an obvious advantage in experiments intended for *e.g.* localizing specific components *in situ* or tracking labelled molecules or nanoparticles. Conventional processing with aldehyde-osmium fixation and Epon embedding was applied since these samples allow a refined high-resolution visualization of all tissue/cell components, thus providing unequivocal information on their structural preservation.

Soleus muscle was selected due to its small size, allowing rapid diffusion of fluids and solutes from and to the culture medium; moreover, soleus can easily be isolated and dissected out without damaging the muscle belly since it originates from the head of the fibula by a slender tendon, and inserts onto the tuber calcanei (together with the gastrocnemius muscle) by the Achilles' tendon.

Control muscles where characterized by myofibres with their distinctive banding pattern and multiple myonuclei located in subsarcolemmal position (Figure 2). In detail, the sarcoplasm was mostly occupied by the longitudinally arrayed myofibrils, with myofilaments arranged in sarcomeres; ovoid mitochondria, membrane structures belonging to the sarcoplasmic

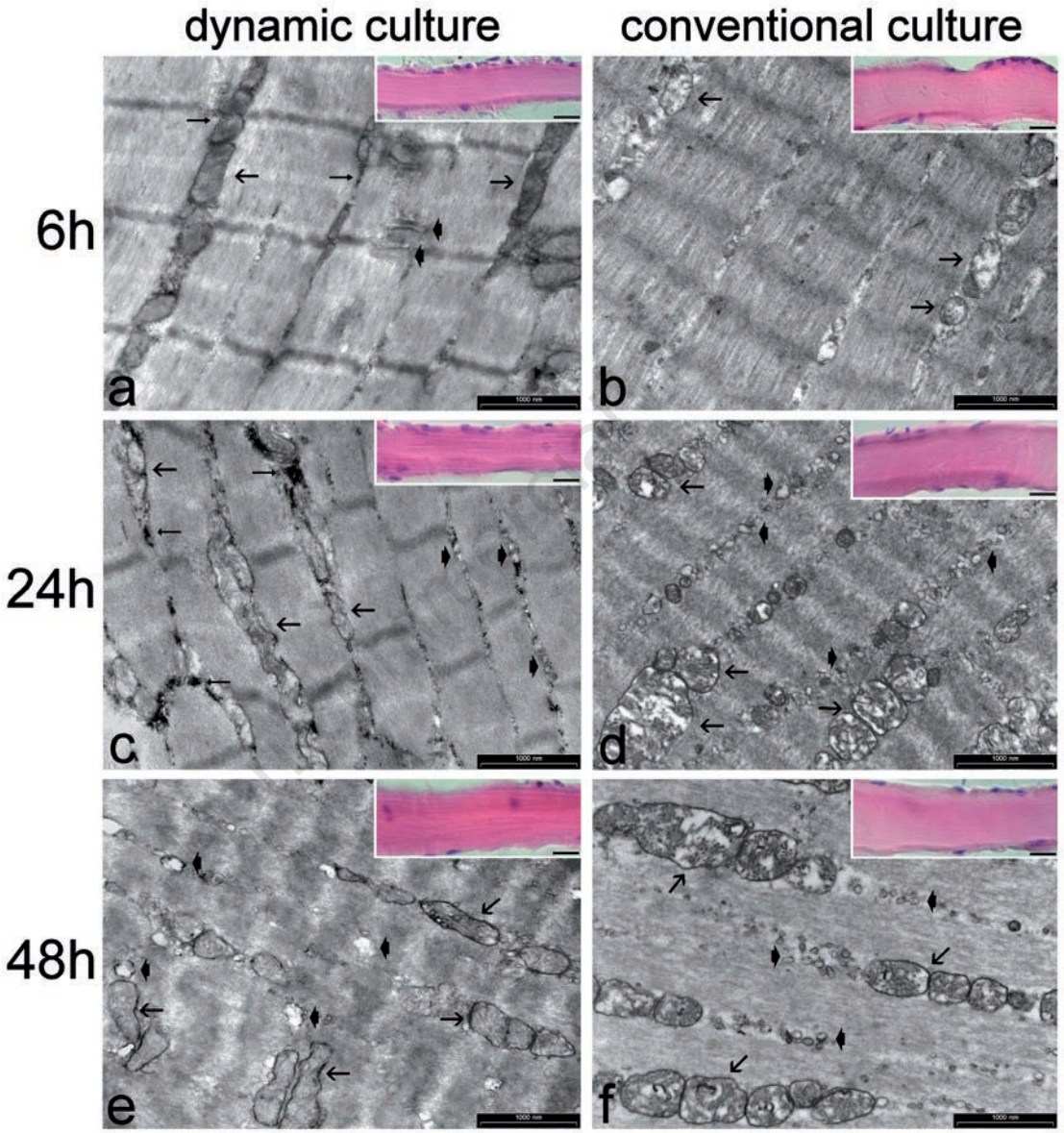

Figure 3. Soleus muscle, samples maintained in culture under fluid dynamic (a,c,e) or conventional (b,d,f) conditions. After 6 h incubation in the bioreactor (a), sarcomere organization is preserved, as well as mitochondria (arrows), sarcoplasmic reticulum (arrowheads) and glycogen (thin arrows); under conventional culture condition (b), cytoskeleton is loosened, mitochondria (arrows) are swollen and glycogen is quite scarce. After 24 h incubation in the bioreactor (c), cytoskeletal organization is still recognizable, and mitochondria (arrows), sarcoplasmic reticulum (arrowheads) and glycogen (thin arrows) are well preserved; under conventional condition (d) the cytoskeletal architecture is loosened, mitochondria (arrows) and sarcoplasmic reticulum (arrowheads) are swollen and glycogen is lost. After 48 h in the bioreactor (e), sarcomere organization is hardly recognizable, sarcoplasmic reticulum (arrowheads) is swollen, but mitochondria (arrows) are still preserved; under conventional condition (f), the cytoskeletal organization is definitely lost, and mitochondria (arrows) and sarcoplasmic reticulum (arrowheads) are strongly damaged. Scale bars: 1000 nm. Insets scale bars: 10 µm.

reticulum and glycogen granules occurred between the myofibrils (Figure 2a); the myonuclei showed small condensed chromatin clumps at both the nuclear and nucleolar periphery, and one or two roundish nucleoli (Figure 2b).

Explanted muscles maintained under *in vitro* conditions underwent a progressive structural deterioration during their permanence in the culture medium; however, both light and electron microscopy observations demonstrated that fluid dynamic conditions markedly slowed this process (Figure 3).

After 6 h in the bioreactor, the general appearance of the muscle tissue was almost comparable to the one of samples immediately fixed after dissection (Figure 3a): in the sarcoplasm, the spatial arrangement of myofilaments and the sarcomere architecture were clearly recognizable, ovoid shaped mitochondria with well-preserved cristae were lined between the myofibrils, the sarcoplasmic reticulum did not show any enlargement, and glycogen clusters were present; myonuclei did not show morphological alterations (*not shown*). On the contrary, after 6 h under conventional condition, the cytoskeletal organization showed some loosening and mitochondria were often swollen; moreover, glycogen deposits were very scarce (Figure 3b). It is known that glycogen is markedly affected by *post mortem* processes, undergoing significant decrease, de-location and even disappearance.[4,5] In myonuclei no structural alteration was observed (*not shown*).

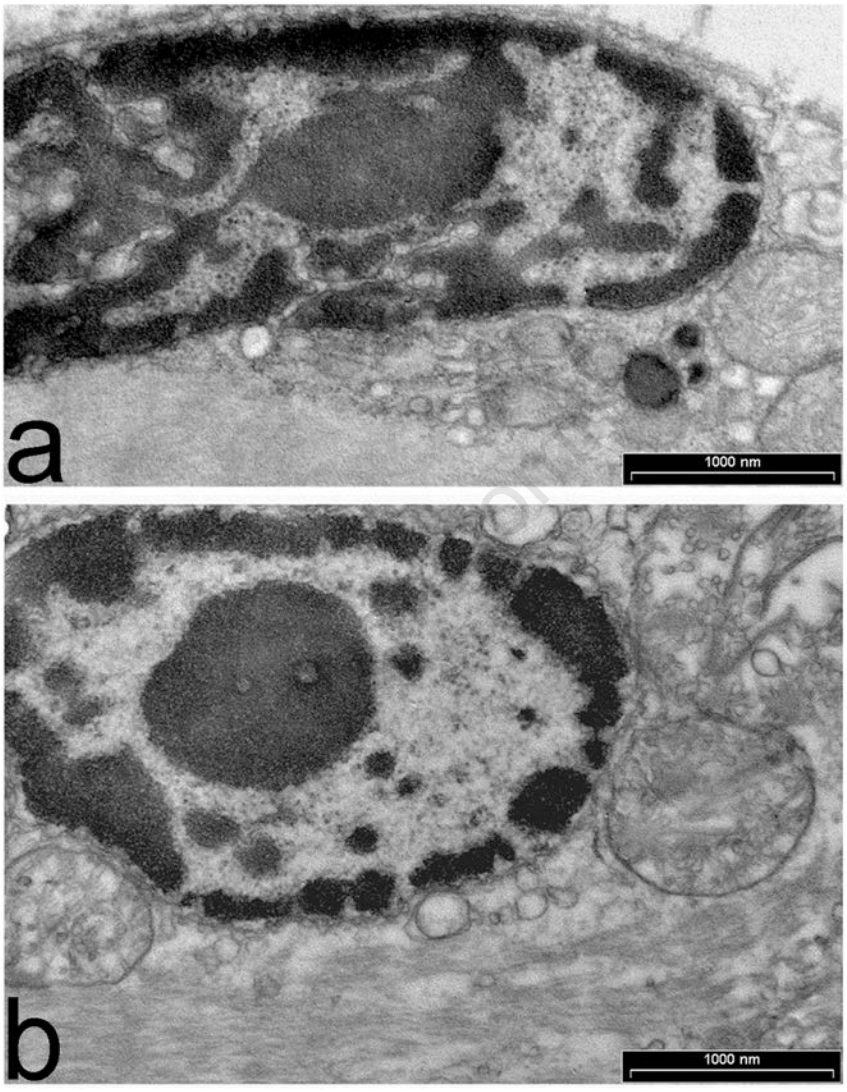

Figure 4. Soleus muscle; samples maintained in culture for 48 h under fluid dynamic (a) or conventional (b) conditions. Both myonuclei show clumps of condensed chromatin at their periphery and one condensed nucleolus. In conventional conditions (b), the nuclear envelope is wrinkled and the perinuclear space is enlarged.

After 24 h of permanence in culture, the difference in structural preservation became even more marked: under fluid dynamic conditions, the histological and ultrastructural features of the muscle were still preserved and detectable, despite some loosening (Figure 3c), whereas muscles cultured under conventional conditions underwent dramatic cytological damage, with sarcomere disorganization, swelling of the mitochondria and sarcoplasmic structures, and massive glycogen loss (Figure 3d), although cell nuclei still preserved a good morphology (*not shown*).

After 48 h, the muscles maintained under fluid dynamic conditions underwent important cytoskeletal disorganization, although some sarcomere remnants were still appreciable even at light microscopy (Figure 3e); the sarcoplasmic reticulum underwent swelling and glycogen was lost, while most of the mitochondria maintained their typical morphology. Myonuclei seemed to undergo some condensation of the peripheral chromatin, but both nucleoli and nucleoplasmic structural constituents maintained their usual morphological features, and no evidence of necrosis or apoptosis was observed (Figure 4a). The muscles cultured under conventional condition definitely lost the ordered myofibril arrangement, while the cytoplasmic organelles underwent morphological alterations typical of necrosis (Figure 3f). In myonuclei, chromatin condensation was more evident than in samples maintained under fluid dynamic conditions, the nuclear envelope appeared as wrinkled and the perinuclear space as enlarged (Figure 4b); in some cases, massive autolytic artefacts were observed (*not shown*).

These results, obtained by both light and transmission electron microscopy, demonstrate that fluid dynamic culture is suitable to prolong the structural preservation of explanted skeletal muscle in comparison to conventional conditions. The bioreactor used in the present study was found to stimulate metabolism in hepatocytes, epithelial cells and adipocytes,[6-8] and to ameliorate permeability in cultured enterocytes,[9,10] in both 2D and 3D cultures. The metabolism of skeletal muscles is known to be especially sensitive to variations in flow rate,[11] and the fluid dynamic environment probably stimulates cell activity by facilitating molecular turnover through an increased oxygen/nutrient supply and faster catabolite removal.

Interestingly, myofibrillar organization seems to be more affected by the culture condition than the mitochondria, although these organelles are known to be highly sen-

sitive to environmental alterations. It is likely that the structural preservation of the sarcomeric arrangement is related also to mechanoreceptor stimulation[12,13] which was obviously lost in the bioreactor too.

Previously reported culture methods of isolated skeletal muscles were based on the incubation in a shaking water bath at 35°C with O_2/CO_2 insufflation: the muscles were maintained under such conditions until 18 h and then submitted to biochemical analysis.[3,14-17] The bioreactor used for the present study seems to be more efficient as it may be directly placed inside the incubator, thus allowing to maintain steady levels of temperature, humidity, O_2 and CO_2, which are essential factors to improve cell survival under *in vitro* conditions. In addition, this fluid dynamic system may ensure a culture environment more similar to the physiological one than other previously proposed incubation methods: in our experiment, the flow rate in the bioreactor was set at 300 µL/min, to reproduce the blood flow values in skeletal muscle,[18] but the fluid flow may be finely modulated, to mimic metabolic changes for functional studies. We cannot exclude that an increase in the flow rate could further ameliorate muscle preservation by improving metabolite/ catabolite turnover. A concomitant temperature decrease of a few degrees would possibly prolong the *in vitro* preservation, but this would obviously make the tissue differently responsive to the experimental stimuli.

It is worth noting that the availability of reliable systems for organ preservation and culture will have a positive ethical and economic impact on research activities allowing to effectively reduce the experimentation on animals. Prolonging the preservation of explanted organs under *in vitro* conditions expands their potential as experimental systems suitable for basic research as well as for efficacy/safety tests on chemicals, pharmaceuticals, nanocomposites and food/feed components.

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